

77.11.00

SL Box



McGREGOR & ADLER, LLP

P.O. BOX 710509
HOUSTON, TEXAS 77271-0509



WRITER'S DIRECT DIAL
(713)-777-2321
WRITER'S DIRECT FACS.
(713)-777-6908
E. MAIL: badler1@houston.rr.com

INTELLECTUAL PROPERTY LAW
(PATENT, BIOTECHNOLOGY, COMPUTER,
TRADEMARK & TRADE SECRET LAW)

Docket No.: D6070CIP

The Assistant Commissioner of Patents And Trademarks
BOX PATENT APPLICATION
Washington, DC 20231

Dear Sir:

Transmitted herewith for filing is the non-provisional patent in the:

Name of: **Curiel & Krasnykh**
For: ***Modified Adenovirus Containing a Fiber Replacement Protein***

CLAIMS AS FILED

| Fee for: | Small entity | Amount |
|--|--------------|--------|
| Basic fee | \$ 345 | \$ 345 |
| Each independent claim in excess of 3 (0) | | |
| Each claim excess of 20 (0) | | |
| Multiple dependent claim | | |
| TOTAL FILING FEE | | \$ 345 |

☒ Enclosed is check for \$345

___ Please charge my Deposit Account No. _____ in the total
amount of the filing fee and the assignment recordation fee if any.

X The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 07-1185.

X Any additional fees under 37 CFR 1.16.

X Any application processing fees under 37 CFR 1.17.

X Small Entity Statement

A small entity statement is enclosed and its benefit under 37 CFR 1.28(a) is hereby claimed.

X Relate Back--35 U.S.C. 120

This continuation-in-part application claims benefit of U.S. application Serial No. 09/250,580 filed February 16, 1999, which claims benefit of U.S. provisional application Serial No. 60/074,844 filed February 17, 1998, now abandoned.

— Assignment

The application is assigned by the inventors to the UAB Research Foundation. The assignment, Recordation Form Cover Sheet and the \$40 fee is enclosed.

X Sequence Listing

A Sequence Listing along with a Sequence Compliance Statement and a computer readable form of the Sequence Listing is enclosed.

X Power of Attorney

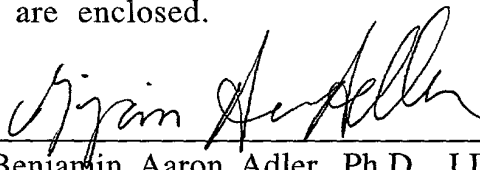
X is attached.

X Address all future communications to:

Benjamin Aaron Adler
McGREGOR & ADLER, LLP.
8011 Candle Lane,
Houston TX 77071
(713) 777-2321

X Two photocopies of this sheet are enclosed.

Date: 7/10/00



Benjamin Aaron Adler, Ph.D., J.D.
Agent for Applicant
Registration No. 35,423

07/10/00
JCS42 U.S. PTO

07-11-00

H

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Curiel & Krasnykh

§
§
§
§
§
§

GROUP ART UNIT:

FILED: July 10, 2000

EXAMINER:

SERIAL NO.:

DOCKET: D6070CIP

FOR: Modified Adenovirus Containing
A Fiber Replacement Protein

JCS42 U.S. PTO
09/612852

07/10/00

CERTIFICATE OF MAILING BY EXPRESS MAIL UNDER 37 CFR 1.10

Assistant Commissioner of Patents and Trademarks
BOX NON-PROVISIONAL PATENT APPLICATION
Washington, D.C. 20231

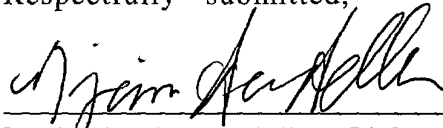
Dear Sir:

I hereby certify that the following documents, which are attached, are being deposited, under 37 CFR 1.10, with the United States Postal Service "Express Mail Post Office to Addressee" service as Express Mail No. EL559421302US in an envelope addressed to: The Assistant Commissioner of Patents and Trademarks, Washington, D.C. 20231, BOX NON-PROVISIONAL PATENT APPLICATION.

- 1) Continuation-in-part application including 48 pages of specification, 4 pages of claims and 14 sheets of drawings;
- 2) Sequence Listing, Compliance Statement & CRF disk
- 3) Transmittal letter;
- 4) Small Entity Status Form;
- 5) (2) Two Combined Declarations and Power of Attorney;
- 6) Filing fee (\$345) and return postcard.

Please return the enclosed postcard acknowledging receipt of this correspondence.

Respectfully submitted,



Benjamin Aaron Adler, Ph.D., J.D.
Registration No. 35,423

Date: 7/10/00

McGREGOR & ADLER, LLP
8011 Candle Lane
Houston, Texas 77071
(713) 777-2321

Applicant or Patentee: Curiel & Krasnykh Attorney's 6070CIP
Serial or Patent No.: _____ Docket No.: _____
Filed or Issued: July 10, 2000
For: Modified Adenovirus Containing a Fiber Replacement Protein

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY
STATUS (37 CFR 1.9(f) and 1.27(c)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official of the nonprofit organization empowered to act on behalf of the concern identified below:

NAME OF ORGANIZATION University of Alabama at Birmingham Research Foundation
ADDRESS OF CONCERN 701 20th Street South, Birmingham, AL 35294-0011
☒ X University or other institution of higher education

I hereby declare that the above identified nonprofit organization qualifies as a nonprofit organization as defined in 37 CFR 1.9(e), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization identified above with regard to the invention, entitled as above

_____ by inventor(s) as above
described in:

- ☒ the specification filed herewith
☐ application serial no. _____, filed
☐ patent no. _____, issued

If the rights held by the above identified nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME

ADDRESS

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING David Day

TITLE OF PERSON OTHER THAN OWNER Director

SIGNATURE David L. Day

DATE 7-10-2000

**MODIFIED ADENOVIRUS CONTAINING A FIBER
REPLACEMENT PROTEIN**

5

10

BACKGROUND OF THE INVENTION

Cross-Reference to Related Applications

This continuation-in-part application claims benefit
of U.S. application Serial No. 09/250,580 filed February 16, 1999,
15 which claims benefit of U.S. provisional application Serial No.
60/074,844 filed February 17, 1998, now abandoned.

Federal Funding Legend

This invention was supported in part using federal funds
20 from the National Institutes of Health. Accordingly, the Federal
Government has certain rights in this invention.

Field of the Invention

The present invention relates generally to the fields of vector biology and gene therapy. More specifically, the present invention relates to the production of recombinant adenoviral vectors with replacement of fibers for cell-specific targeting with concomitant elimination of endogenous tropism.

Description of the Related Art

10 Approaches to target adenoviral vectors to specific cell types should be based on an understanding of the mechanism of cell entry exploited by the majority of human adenoviruses and on the identification of the components of the adenoviral virion which are involved in the early steps of the virus-cell interaction.

15 Adenoviruses are non-enveloped viruses containing a double stranded DNA genome packaged into an icosahedral capsid. Whereas the most abundant capsid protein, the hexon, performs structural functions and is not involved in the active cell entry process, the other two major protein components of the capsid, the fiber and the

20 penton base, have been shown to play key roles in the early steps of virus-cell interaction. The fiber and penton base together form

penton capsomers consisting of five penton base subunits embedded in the virus capsid tightly associated with a homotrimer of fiber proteins protruding from the virion.

Each of the five subunits of the penton base contains a flexible loop structure, which corresponds to a hypervariable domain of the otherwise highly conserved protein. Amino acid sequence analysis of penton base proteins of different adenoviral serotypes showed that each loop consists of two stretches of alpha helices flanking an arginine-glycine-aspartic acid (RGD) tripeptide positioned in the middle of the loop. Cryo-electron micrography (cryo-EM) studies of Ad2 virions revealed that these loops form 22 Å protrusions on the surface of penton base, thereby facilitating interaction of the RGD motif, localized at the apex of the protrusion with cellular integrins.

The fiber has a well-defined structural organization with each of its three domains, the tail, the shaft, and the knob, performing a number of functions vital for the virus. The short amino terminal tail domain (46 amino acid residues in Ad2 and Ad5 fibers) of the fiber protein is highly conserved among most adenoviral serotypes. In addition to being involved in the association with the penton base protein through an FNPVYD motif at residues

11-16, which results in anchoring the fiber to the adenoviral capsid, the tail domain also contains near its amino terminus the nuclear localization signal KR λ R (where λ indicates a small amino acid residue), which directs the intracellular trafficking of newly synthesized fibers to the cell nucleus, where the assembly of the adenoviral particle takes place.

The central domain of the fiber is the shaft, which extends the carboxy terminal knob domain away from the virion, thereby providing optimal conditions for receptor binding. The shaft is organized as a sequence of pseudorepeats, each 15 amino acids in length, with a characteristic consensus sequence containing hydrophobic residues at highly conserved positions. This sequence, X-X- ϕ -X- ϕ -X- ϕ -G-X-G- ϕ -X- ϕ -X-X or X-X- ϕ -X- ϕ -X- ϕ -X-X-P- ϕ -X- ϕ -X-X, contains hydrophobic amino acids at “ ϕ ”-positions, with either the eighth and tenth positions being occupied with two glycines or with a proline in the tenth position. The models for the secondary structure corresponding to these repeats describe the shaft as a triple β -spiral in which the β -strands are oriented more along the fiber axis and the hydrophobic residues at the 7th and 13th position are located at greater radius. The trimer is stabilized with extensive intra- and

inter-chain hydrogen bonding. Due to its rod-like shape, the shaft domain basically determines the length of the entire molecule, which depends on the number of pseudorepeats contained within the shaft. The fibers of various human adenoviral serotypes contain different
5 number of repeats, resulting in a significant variation in the fiber length: from 160Å (Ad3) to 373Å (Ad2 and Ad5).

The carboxy terminal knob domain (180-225 amino acid residues) carries out two distinct functions, i.e., initiation of fiber trimerization and binding of the virus to its primary cellular
10 receptor. X-ray crystallography studies on *E.coli*-expressed Ad5 fiber knob protein have shown that the trimeric knob is arranged around a three-fold crystallographic symmetry axis and resembles a three bladed propeller when viewed along this axis. Each monomer of the knob is a β -sandwich structure, formed by two antiparallel β -
15 sheets R and V. The surface of the V-sheet, which consists of the strands A, B, C, and J, points towards the virion, while the R-sheet, formed by strands D, I, H, and G, points outside the virion and towards the surface of the target cell. These findings have been then corroborated with X-ray crystallography data obtained with
20 recombinant Ad2 fiber knob protein.

A number of studies employing recombinant knobs have shown that these proteins are capable of self-trimerization, which does not require any cellular chaperons. The exact trimerization motif within the fiber knob is largely unknown, which makes
5 mutagenesis or modification of this protein quite difficult: indeed, any new mutation or modification of the fiber may affect amino acid(s) involved in the fiber trimerization and may therefore destabilize the entire molecule, thereby rendering it non-functional. The mutant knobs revealed that deletions in the knob sequence,
10 even as short as two amino acid residues, may result in monomeric fibers, which cannot associate with penton base and, therefore, cannot be incorporated into mature adenoviral particles.

The second function performed by the knob is binding to a cellular receptor and, therefore, mediating the very first step of the
15 virus-cell interaction. This receptor-binding ability of the knob has been demonstrated by utilization of recombinant knob proteins as specific inhibitors of adenoviral binding to cells. Based on the β -sandwich structure of the knob, it was originally hypothesized by Xia et al. that the strands constituting the R-sheet form a receptor
20 binding structure. Recently, however, analysis of fiber knob mutants has revealed that segments outside the R-sheet constitute the

receptor-binding site. The Ad5 binding site is located at the side of the knob monomer and specifically involves sequences within the AB and DE loops and B, E, and F β -strands. The binding site of Ad37 that binds to a different receptor involves a critical residue in the CD loop
5 at the apex of the trimer.

The two penton proteins, the penton base and fiber, work in a well-orchestrated manner to provide the early steps of the cell infection mechanism developed by adenoviruses. Importantly, each of these early events is mediated by either fiber or penton base;
10 therefore, both proteins play distinct and well defined roles in this process.

The fiber knob provides the initial high-affinity binding of the virus to its cognate cell surface receptor, coxsackievirus and adenovirus receptor (CAR), which does not possess any
15 internalization functions and merely works as a docking site for Ad attachment.

Human adenoviruses (Ad) of serotype 2 and 5 have been extensively used for a variety of gene therapy applications. This is largely due to the ability of these vectors to efficiently deliver
20 therapeutic genes to a wide range of different cell types. However, the promiscuous tropism of adenovirus resulting from the

widespread distribution of coxsackie virus and adenovirus receptor (CAR) (1, 2), limits the utility of adenoviral vectors in those clinical contexts where selective delivery of therapeutic transgene to a diseased tissue is required. Uncontrolled transduction of normal
5 tissues with adenoviral vectors expressing potentially toxic gene products may lead to a series of side effects, thereby undermining the efficacy of the therapy. Furthermore, cell targets expressing CAR below certain threshold levels are not susceptible to adenoviral-based therapies due to their inability to support adenoviral infection.
10 Therefore, the dependence of the efficiency of the adenoviral-mediated cell transduction on the levels of CAR expression by the target cell presents a serious challenge for the further development of adenoviral-based gene therapeutics.

In order to overcome this limitation, the concept of
15 genetic targeting of adenoviral vectors to specific cell surface receptors has been proposed. Strategies to retarget adenoviral vectors are based on the currently accepted model of adenoviral infection (3), which postulates that the initial binding of the adenoviral virion to the cell is mediated by the attachment of the
20 globular knob domain of the adenoviral fiber protein to CAR. This is then followed by an internalization step triggered by the interaction

of the RGD-containing loop of a second adenoviral capsid protein, the penton base, with cellular integrins. Although recent studies have shown that representatives of different adenoviral serotypes may utilize cell receptors other than CAR, the two-step mechanism of cell entry established for Ad2 and Ad5 appears to be common to the majority of human adenovirus. As the fiber protein is the key mediator of the cell attachment pathway employed by Ad, genetic incorporation of targeting ligands within this viral protein was originally proposed as the strategy to derive targeted, cell type specific adenoviral vectors.

Although the primary amino acid sequences of fiber proteins of various human and animal adenoviruses are highly diverse, the overall structural and functional organization of these proteins demonstrate remarkable degree of similarity. Indeed, all key features of the domains of the fiber proteins described above - the presence of the nuclear localization signal and the penton base binding site within the fiber tail; the presence of pseudorepeats in the shaft; the propeller-like structure of the knob; and trimeric configuration of the entire fiber molecule - are highly conserved between various adenoviral serotypes. This overall structural and functional similarity has been exploited by a number of

investigators, who succeeded in replacing the entire fiber proteins of one adenoviral serotype with those derived from another serotype, or “shuffled” individual domains of the fiber molecule utilizing a variety of structural domains pre-existing in nature.

5 However, it is of paramount importance to note that fiber shuffling does not overcome the limitations associated with the conserved structure of native fibers: as all the adenoviral fibers characterized so far contain the knob domains of similar structure, which carry out the functions of trimerization and receptor binding,
10 it is logical to assume that replacing those knobs with their structurally similar counterparts derived from other adenoviral serotypes would lead to chimeric molecules inheriting all the drawbacks and structural limitations known for the wild type fibers in the context of incorporation of the cell-targeting ligands within
15 these carrier proteins. The same holds true with respect to shuffling of the full size fibers.

 In addition, as all wild type adenoviral fibers have affinity to their cognate receptors, it is rather problematic to create recombinant adenoviral vectors targeted to specific cell surface
20 receptors via the fiber shuffling. This maneuver may change the tropism of the vector, but will never result in an adenoviral vector

specifically targeted to the cell of interest. Although ablation of native tropism of adenoviral vector via identification and subsequent elimination of specific amino acids of the fiber protein which mediate binding of the virion to its native receptor is generally viewed as the way of derivation of truly targeted adenoviral vectors, it may have limited utility as the mutated sequences may undergo reversion to the wild type during multiple cycles of virus propagation. Due to its restored ability to bind to its native receptor a virion which genome underwent such a reversion immediately achieves selective advantage over the virions which tropism is restricted to one specific receptor. This selective advantage will eventually result in significant contamination of the vector preparation with virions retaining tropism to receptors different from the target one. Therefore the efficiency of the entire targeting maneuver will be jeopardized.

Furthermore, many human adenoviruses recognize CAR as the primary binding receptor which is expressed by many different cell types. Taken together with the widespread distribution of adenoviral infections in humans, this has led to the belief that chimeric adenoviral virions incorporating fiber proteins originating from different adenoviral serotypes most likely exist in nature when

the same cell in a human body gets infected with two adenoviruses belonging to two different Ad serotypes. Therefore, shuffling the fibers is an experimental realization of the viral chimerism which takes place naturally.

5 Attempts to generate adenoviral vectors possessing expanded tropism involved incorporation of short peptide ligands into either the carboxy terminal or so-called HI loop of the knob of the Ad fiber protein. Although these studies demonstrated the feasibility of genetic targeting of Ad and showed the potential utility
10 of such vectors in the context of several disease models (7, 8), further progress in this direction has been hampered by the structural conflicts often observed as a result of modification of the fiber structure. Due to the rather complex structure of the fiber knob domain, even minor modifications to this portion of the
15 molecule may destabilize the fiber, thereby rendering it incapable of trimerization and, hence, non-functional. The upper size limit for a targeting ligand to be incorporated into Ad5 fiber is about 30 amino acid residues (5, 9), which dramatically narrows the repertoire of targeting moieties, thereby limiting the choice of potential ligands
20 and, therefore, cell targets. The task of adenoviral targeting is further complicated by the need to ablate the native receptor-

binding sites within the fiber of an adenoviral vector to make it truly targeted. As a result of these limitations, only a handful of heterologous peptide ligands (oligo lysine, FLAG, RGD-4C, RGS(His)₆, and HA epitope) have been successfully used in the context of Ad5 fiber modification during last several years.

The prior art remains deficient in the lack of effective means to produce recombinant adenoviral vectors with combination of novel targeting and ablation of native tropism. The present invention fulfills this longstanding need and desire in the art.

SUMMARY OF THE INVENTION

The present invention describes the next generation of recombinant, cell-specific adenoviral vectors. More particularly, the instant specification discloses that there are two aspects to consider in the modification of adenoviral tropism: (1) ablation of endogenous tropism; and (2) introduction of novel tropism. To expand the utility of recombinant adenoviruses for gene therapy applications, methods

to alter native vector tropism to achieve cell-specific transduction are necessary. To achieve such targeting, the present invention discloses the development of a targeted adenovirus created by radical replacement of the adenovirus fiber protein. The fiber
5 protein was replaced with a heterologous trimerization motif to maintain trimerization of the knobless fiber and a ligand capable of targeting the virion to a novel receptor was introduced simultaneously. The present invention thus represents a demonstration of the retargeting of a recombinant adenoviral vector
10 via a non-adenoviral cellular receptor.

In one embodiment of the present invention, there is provided a recombinant adenovirus vector lacking endogenous viral tropism but having novel tropism. The adenovirus vector is modified to produce a replacement adenoviral fiber protein so as to modify
15 viral tropism, wherein the replaced fiber gene comprises the amino-terminal portion of the adenoviral fiber gene including the tail domain, the carboxy-terminal portion of the T4 bacteriophage fibritin gene and a ligand. The fiber replacing protein retains the fiber's capacity to trimerize. Preferably, the ligand can be a
20 physiological ligand, anti-receptor antibodies or cell-specific

peptides. The adenoviral vector may further contains a therapeutic gene such as the herpes simplex virus-thymidine kinase gene.

In another embodiment of the present invention, there is provided a method of killing tumor cells in an individual in need of
5 such treatment, comprising the steps of pretreating said individual with an effective amount of the recombinant adenoviral vector disclosed herein and administering ganciclovir to said individual.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description
10 of the presently preferred embodiments of the invention given for the purpose of disclosure.

15 **BRIEF DESCRIPTION OF THE DRAWINGS**

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more
20 particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are

illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

5 **Figure 1** shows the generation of Ad5 fiber-T4 fibrin chimera containing targeting ligand. **Figure 1A** shows the schema showing key components of the fiber-fibrin-ligand chimera and their sources. The tail of the fiber anchors the fiber-fibrin-6His chimera in the Ad virion; a fragment of the fibrin protein provides
10 trimerization of the molecule; while the 6His ligand mediates binding to an artificial receptor.

Figure 1B shows SDS-PAGE analysis of *E.coli*-expressed, IMAC-purified FF/6H chimeric protein. M – molecular mass protein ladder (200, 116, 97, 66 and 45 kilodaltons markers are seen), lanes
15 1 and 2 – FF/6H protein, lanes 3 and 4 – wild type Ad5 fiber. Samples in lanes 1 and 3 are denatured by boiling, which resulted in degradation of trimeric proteins to monomers, while lanes 2 and 4 contain proteins in their native trimeric configuration.

Figure 2 shows the structure of the fiber-fibrin-6H
20 protein chimera. The FF/6H gene assembled by overlap extension PCR encodes a 373 amino acid long protein chimera which consists of

the amino terminal segment of Ad5 fiber protein genetically fused with the carboxy terminal portion of the T4 fibrin protein, followed with the linker and the 6His-containing ligand. The beginning of the third pseudorepeat of the fiber shaft domain (GNTLSQNV) (SEQ ID NO. 5 11) is joined to the fibrin sequence starting with the fragment of the insertion loop (SQN) preceding the sixth coiled coil segment of the α -helical central domain of the . fibrin (VYSRLNEIDTKQTTVESDISAIKTSI) (SEQ ID NO. 12). The sequence SQNV present in the native structures of both fusion partners was 10 chosen as the hinge between the two molecules in order to minimize potential structural conflicts between the β -spiral configuration of the fiber shaft and the triple α -helix of the central domain of the fibrin. The segments of the fibrin sequence localized between every two adjacent coiled coils are the insertion loops which provide 15 some degree of flexibility needed for optimal ligand presentation. A peptide linker is incorporated between the carboxy terminal trimerization domain (foldon) of the fibrin and the six histidine containing ligand to extend the ligand away from the carrier protein in order to facilitate binding to the target receptor.

Figure 3 shows the Ad-mediated gene transfer to 293/6H cells. 293/6H cells were derived by transfection of 293 cells with a recombinant plasmid expressing an artificial receptor (AR), which consists of an anti-5His scFv genetically fused with the transmembrane domain of the PDGF receptor. Due to the presence of both CAR and AR on the surface of these cells, 293/6H are susceptible to infection by both the Ad with the wild type fibers and the Ad incorporating the FF/6H chimera. Importantly, each virus is capable of binding to only one type of receptor, CAR or AR. The progenitor cell line, 293, is refractory to Ad5LucFF/6H infection.

Figure 4 shows the generation of Ad5LucFF/6H. The genome of the wild type Ad5 was modified by homologous DNA recombination in *E.coli* to contain a firefly luciferase expressing cassette in place of the E1 region, as well as the gene encoding the FF/6H chimera, which replaced the wild type fiber gene. The virus, Ad5LucFF/6H, was first rescued in 211B cells expressing the wild type Ad5 fiber. The seed stock of the virus obtained at this point contained a mixed population of Ad virions with mosaic capsids incorporating both wild type Ad5 fibers and FF/6H proteins. In order to obtain a homogeneous population of Ad virions containing FF/6H chimeras, this stock was then used to infect 293 cells

expressing the artificial receptor, 293/6H. The virus isolated from 293/6H cells was purified by double banding on a CsCl gradient.

Figure 5 shows the analysis of Ad5LucFF/6H capsid composition. **Figure 5A** shows SDS-PAGE of CsCl-purified

5 Ad5LucFF/6H virions. Samples containing 4×10^{10} particles of either the wild type Ad5 (lane 1) or Ad5LucFF/6H (lane 2) were boiled in Laemmli sample buffer and fractionated on a 10% SDS-PAGE gel. Of note, the resolution of this minigel is not sufficient for separation of the fiber and protein IIIa.

10 **Figure 5B** shows Western blot analysis of FF/6H chimeras incorporated into Ad5LucFF/6H virions. Proteins of denatured Ad5LucFF/6H virions, lane 2, were separated on a 10% SDS-PAGE gel and then probed with anti-Ad fiber tail mAb 4D2, anti-5His mAb Penta-His and anti-fibritin mouse polyclonal antibodies.

15 Wild type Ad5, lane 1, and Ad5LucFc6H, a virus containing fibers with carboxy terminal 6His tags, lane 3, were used as controls.

Figure 6 shows the binding of Ad5LucFF/6H virions to Ni-NTA-agarose. Wild type Ad5 or Ad5LucFF/6H were incubated with an aliquot of Ni-NTA-resin for one hour. The matrix was

20 pelleted by centrifugation and the supernatant was removed and then incubated with a second aliquot of Ni-NTA-agarose. Aliquots of

material subsequently eluted from the resin, as well as an aliquot of the material present in the supernatant after two sequential incubations with the resin, were separated on a 10% SDS-PAGE gel and then stained (**Figure 6A**) or probed with either anti-fiber tail mAb 4D2 (**Figure 6B**) or with anti-5His mAb Penta-His (**Figure 6C**).

Lane 1, aliquot of the virus prior to incubation with Ni-NTA-agarose; lane 2, material bound to the first aliquot of the resin; lane 3, material bound to the second aliquot of the resin; lane 4, material remaining in the supernatant after two sequential bindings to the resin. Incomplete binding of Ad5LucFF/6H virions to Ni-NTA-agarose is most likely due to the small size of pores in the Sepharose CL-6B used as the matrix for manufacturing Ni-NTA-agarose. According to the manufacturer's specifications, the size of those pores does not allow protein molecules with molecular mass larger than 4MDa to enter the pores. Thus, the Ni-NTA groups which are localized on the surface of the Sepharose particles are accessible to the 6His-tagged virions (relatively small percentage), whereas those hidden inside the pores (the majority) are not.

Figure 7 shows the analysis of Ad5LucFF/6H genome structure. **Figure 7A** shows DNA isolated from purified Ad5LucFF/6H virions subjected to restriction enzyme analysis using

a number of restriction endonucleases which do not cleave the wild type fiber gene sequence but cleave the FF/6H gene. Odd-numbered lanes – control Ad5Luc1 DNA; even-numbered lanes – Ad5LucFF/6H DNA. **Figure 7B** shows “diagnostic PCR” utilizing a pair of primers flanking the fiber gene in Ad5 genome employed to show the absence of the wild type fiber gene sequence in the Ad5LucFF/6H genome: lane 1, PCR product amplified from wild type Ad5 DNA; lane 2, PCR product amplified from Ad5LucFF/6H DNA; M – 1Kb ladder.

Figure 8 shows the evaluation of the efficiency and receptor-specificity of Ad5LucFF/6H-mediated gene transfer. **Figure 8A** shows gene transfer to 293 and 293/6H cells. Cells seeded in 24-well plates were infected with various doses of Ad5LucFF/6H. The minimal viral dose corresponding to a multiplicity of infection of 40 viral particles per cell, (1X), was equal to the dose of the control virus, Ad5Luc1, whereas Ad5LucFF/6H doses 10x, 100x, and 1000x contained 10-, 100-, and 1000-times the amount of the control virus, correspondingly. Twenty hours post-infection, the cells were collected, lysed, and the luciferase activity of the lysates was measured in relative light units.

Figure 8B shows the specificity of Ad5LucFF/6H binding to the artificial receptor. 293/6H cells grown in monolayer culture

were pre-incubated with various concentrations of either the truncated form of fibrin or fibrin carrying a carboxy terminal 6His tag, fibrin-6H, prior to infection with Ad5LucFF/6H. Luciferase activities detected in the lysates of infected cells twenty hours post-
5 infection were given as percentages of the activity in the absence of blocking protein. Each data point was set in triplicates and calculated as the mean of three determinations.

Figure 9 shows the schema of key components of the fiber-fibrin-RGD/6His chimera.

10 **Figure 10** shows SDS-PAGE analysis of CsCl-purified Ad5LucFF.RGD/6H virions. Samples of either the wild type Ad5 (lane 2) or Ad5LucFF.RGD/6H (lane 1) were boiled in Laemmli sample buffer and fractionated on a 10% SDS-PAGE gel.

Figure 11 shows Western blot analysis of FF.RGD/6H
15 chimeras incorporated into Ad5LucFF/6H virions. Proteins of denatured Ad5LucFF.RGD/6H virions, lane 2, were separated on a 10% SDS-PAGE gel and then probed with anti-Ad fiber tail mAb 4D2, anti-5His mAb Penta-His and anti-fibrin mouse polyclonal antibodies. Wild type Ad5, lane 3, and Ad5LucFc6H, a virus
20 containing fibers with carboxy terminal 6His tags, lane 1, were used as controls.

Figure 12 shows the binding of Ad5LucFF.RGD/6H virions to Ni-NTA-agarose. Wild type Ad5 or Ad5LucFF.RGD/6H were incubated with an aliquot of Ni-NTA-resin. Aliquots of material subsequently eluted from the resin, as well as an aliquot of the virus
5 prior to incubation with Ni-NTA-agarose, were separated on a 10% SDS-PAGE gel and then stained.

Figure 13 shows restriction enzyme analysis of Ad5LucFF.RGD/6H. DNA isolated from purified Ad5LucFF.RGD/6H virions was subjected to restriction enzyme analysis using a number
10 of restriction endonucleases. Odd-numbered lanes – control Ad5Luc1 DNA; even-numbered lanes – Ad5LucFF.RGD/6H DNA.

Figure 14 shows gene transfer by Ad5LucFF.RGD/6H. Cells seeded in 24-well plates were infected with various doses of Ad5LucFF.RGD/6H. The minimal viral dose corresponding to a
15 multiplicity of infection of 40 viral particles per cell, (1X), was equal to the dose of the control virus, Ad5Luc1, whereas Ad5LucFF.RGD/6H doses 10x, 100x, and 1000x contained 10-, 100-, and 1000-times the amount of the control virus, correspondingly. Twenty hours post-infection, the cells were collected, lysed, and the luciferase activity of
20 the lysates was measured in relative light units. _

DETAILED DESCRIPTION OF THE INVENTION

5 In marked contrast to the strategy of replacing one Ad fiber (or one of its domains) with the fiber (or its domain) derived from a different Ad serotype, the present invention presents an alternative approach of Ad targeting based on replacement of the native fiber in an Ad capsid with a chimeric protein, rationally
10 designed to result in permanent ablation of native Ad receptor tropism and simultaneously offers unprecedented flexibility in the generation of novel vector tropism. This work was driven by the hypothesis that these goals may be achieved by “splitting“ the functions normally performed by the knob domain of the Ad5 fiber
15 between two different protein moieties which would replace the knob. Specifically, the knob of the fiber was replaced with a heterologous trimerization motif to maintain trimerization of the knobless fiber and a ligand capable of targeting the virion to a novel receptor was introduced simultaneously. Therefore, in marked
20 contrast to the previous, mostly unsuccessful, attempts to fit a desired ligand into the highly complex framework of the fiber knob

domain, the present invention employs a radical replacement of the fiber with a protein chimera, which allows for utilization of a virtually unlimited range of targeting protein ligands in the context of Ad vector system.

5 The present invention is directed to vector system that provides both a highly efficient and specific targeting of adenovirus vector for the purpose of *in vivo* gene delivery to predefined cell types after administration. In the recombinant adenovirus of the present invention, the adenovirus is modified by replacing the
10 adenovirus fiber protein with a fiber replacement protein. In a preferred embodiment, the fiber replacement protein comprises: a) an amino-terminal portion comprising an adenoviral fiber tail domain; b) a chimeric fiber replacement protein; and c) a carboxy-terminal portion comprising a targeting ligand. A person having
15 ordinary skill in this art would recognize that one may exploit a wide variety of genes encoding e.g. receptor ligands or antibody fragments which specifically recognize cell surface proteins unique to a particular cell type to be targeted.

 The following description will allow a person having
20 ordinary skill in this art to determine whether a putative fiber replacement protein would function as is desired in the compositions

and methods of the present invention. Generally, the fiber replacement protein associates with the penton base of the adenovirus. To prevent problems of incompatibility, the amino-terminus of the chimeric protein can be genetically fused with the
5 tail domain of the adenovirus fiber. Structurally, the fiber replacement protein is preferably a rod-like, trimeric protein. It is desirable for the diameter of the rod-like, trimeric protein to be comparable to the native fiber protein of wild type adenovirus. It is important that the fiber replacement protein retain trimerism when
10 a sequence encoding a targeting ligand is incorporated into the carboxy-terminus. In a preferred aspect, a representative example of a fiber replacement protein is T4 bacteriophage fibritin protein. More generally, the fiber replacement protein can be any native or chimeric protein which is capable of associating with the Ad5 penton
15 base protein and bind to specific cell surface receptor. Other Representative examples of fiber replacement proteins include isoleucine trimerization motif and neck region peptide from human lung surfactant D. Preferably, the fiber replacement protein has a coiled coil secondary structure. The secondary structure provides
20 stability because of multiple interchain interactions. _

In one embodiment, the fiber-replacing molecule engineered in this study incorporated the tail and two amino terminal repeats of the shaft domain of the Ad5 fiber protein genetically fused with a truncated form of the bacteriophage T4
5 fibrin protein, which was employed as the heterologous trimerizing motif in order to compensate for the knob deletion (Fig. 1A). The choice of the T4 fibrin as the trimerization moiety was dictated by a number of its structural features. The fibrin protein is a product of the *wac* gene which forms the “collar” and the “whiskers” of the T4
10 capsid, where it mediates assembly of the long tail fibers and their subsequent attachment to the tail baseplate. Trimerization of this rod-like, 486 amino acid long protein is initiated and maintained by the short (30 aa long) carboxy terminal domain or “foldon”, which is stabilized by a number of hydrophobic interactions and hydrogen
15 bonds (10). The central α -helical domain of fibrin, which consists of 13 segments of parallel triple coiled-coils separated by flexible loop structures, passively follows the trimerization initiated at the carboxy terminal of the molecule. The trimeric structure of fibrin is extremely stable and is not compromised by either extensive amino
20 terminal deletions (up to 92% of the molecule) (11) or carboxy terminal insertions up to, at least, 163 aa long (11, 12). For the

purposes of this study, no receptor-binding function has been shown for fibrin.

In order to provide a receptor-binding ligand, a carboxy terminal six-histidine sequence was connected to the fibrin protein of this fiber-fibrin chimera via a short peptide linker (Fig. 2). The purpose of this maneuver was to demonstrate the feasibility of targeting of fibrin-containing Ad vectors to alternative cell-surface receptors by directing the modified vector to an artificial receptor, which is expressed on the surface of 293/6H cells (Fig. 3). The extracellular domain of this artificial receptor (AR) is an anti-5His single chain antibody, which is genetically fused with the transmembrane domain of the platelet derived growth factor receptor (13). In addition to receptor binding, this 6His sequence was employed to facilitate the detection and purification of the FF/6H chimeras and Ad virions incorporating this protein.

In the adenovirus of the present invention, the targeting ligand is selected from the group consisting of physiological ligands, anti-receptor antibodies, cell-specific peptides and single chain antibodies. In one embodiment, the adenovirus carries in its genome a therapeutic gene. A representative example of a therapeutic gene is a herpes simplex virus thymidine kinase gene.

The present invention is also directed to a method of killing tumor cells in an individual in need of such treatment, comprising the steps of: pretreating said individual with an effective amount of the adenovirus of the present invention; and
5 administering ganciclovir to said individual.

In accordance with the present invention, there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis,
10 Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription and Translation" [B.D. Hames & S.J. Higgins eds.
15 (1984)]; "Animal Cell Culture" [R.I. Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984). Therefore, if appearing herein, the following terms shall have the definitions set out below.

As used herein, "chimera" or "chimeric" refers to a
20 single polypeptide possessing multiple components, often but not necessarily from different organisms. As used herein, "chimeric" is

used to refer to tandemly arranged protein moieties that have been genetically engineered to result in a fusion protein possessing regions corresponding to the functions or activities of the individual protein moieties.

5 As used herein, the terms "fiber gene" refer to the gene encoding the adenovirus fiber protein. As used herein, "chimeric fiber protein" refers to a modified fiber as defined above.

A "fiber replacement protein" is a protein that substitutes for fiber and provide three essential features: trimerizes like fiber,
10 lacks adenoviral tropism and has novel tropism.

As used herein the term "physiologic ligand" refers to a ligand for a cell surface receptor.

In addition, the invention may includes_ portions or fragments of the fiber or fibrin proteins. As used herein,
15 "fragment" or "portion" as applied to a protein or a polypeptide, will ordinarily be at least 10 residues, more typically at least 20 residues, and preferably at least 30 (e.g., 50) residues in length, but less than the entire, intact sequence. Fragments of these genes can be generated by methods known to those skilled in the art, e.g., by
20 restriction digestion of naturally occurring or recombinant fiber or fibrin genes, by recombinant DNA techniques using a vector that

encodes a defined fragment of the fiber or fibrin gene, or by chemical synthesis.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1

10 Construction of the fiber-fibrin-6His (FF/6H) chimera

Generation of the gene encoding the fiber-fibrin-6His chimera was done in several steps. First, a segment of the fibrin gene was PCR-amplified and used to substitute most of the fiber gene sequence encoding the shaft domain. For this, a portion of the T4 fibrin gene encoding the sixth coiled coil through the C-terminal of the protein was amplified with a pair of primers "FF.F" (GGG AAC TTG ACC TCA CAG AAC GTT TAT AGT CGT TTA AAT G) (SEQ ID NO. 1) and "FF.R" (AGG CCA TGG CCA ATT TTT GCC GGC GAT AAA AAG GTA G) (SEQ ID NO. 2). The product of this PCR encodes a segment of an open reading frame (ORF) containing four amino terminal (GLNT) and three carboxy terminal (KIG) codons of the fiber shaft sequence

fused to the fibritin sequence. The reverse primer introduces a silent mutation at the 3' end of the fibritin open reading frame resulting in generation of a unique NaeI-site. Also, NcoI-site was incorporated in the "FF.F" in order to fuse the open reading frame of the fiber and the fibritin. The product of the PCR was then cleaved with NcoI and cloned in the fiber shuttle vector pNEB.PK3.6 (22) cut with NaeI and NcoI. As a result of this cloning, an original NaeI-site in the fiber open reading frame was destroyed, therefore NaeI-site at the end of the fibritin open reading frame remains unique. The plasmid generated was named pNEB.PK.FF_{BB}. This fusion procedure resulted in an open reading frame, in which the fiber and the fibritin sequence were joined via an SQNV peptide hinge, present at the beginning of the 3rd repeat of Ad fiber shaft as well as at the 6th coil coiled segment of the fibritin.

At the next step, a portion of 3' terminal sequence of FF_{BB} open reading frame was replaced with synthetic oligo duplex in order to introduce in the construct a unique restriction site, SwaI, which would allow modifications of the 3' end of the gene. To reach this end, a duplex made of oligos "F5. Δ3Swa.T" (TTG GCC CCA TTT AAA TGA ATC GTT TGT GTT ATG TTT CAA CGT GTT TAT TTT TC) (SEQ ID NO. 3) and "F5. Δ3.Swa.B" (AAT TGA AAA ATA AAC ACG TTG AAA

CAT AAC ACA AAC GAT TCA TTT AAA TGG GGC CAA TAT T) (SEQ ID NO. 4) was cloned in BstXI-MfeI-digested pNEB.PK3.6, thereby generating pNEB.PK Δ3.

To facilitate the downstream manipulation with the 3' end of the fiber-fibritin gene a plasmid pNEB.PK.FF_{BB}Δ3 was generated as follows: an NcoI-Acc65.I-fragment in pNEB.PK.FF_{BB} was replaced with an NcoI-Acc65.I-fragment from pNEB.PKΔ3.

The plasmid pXK.FF_{BB}Δ3 was obtained from pNEB.PK.FF_{BB}Δ3 by deleting a XbaI-fragment containing a portion of the Ad5 Luc-3 DNA. This was done in order to eliminate a BamHI-site contained in this XbaI fragment, which would otherwise compromise the utility of the BamHI-site introduced into the construct at a later step (see below).

To add the sequence encoding a C-terminal linker to the fiber/fibritin fusion protein, a synthetic oligo duplex consisting of oligos “FF_{BB}LL.T” (GGC AGG TGG AGG CGG TTC AGG CGG AGG TGG CTC TGG CGG TGG CGG ATC CGG GGA TTT) (SEQ ID NO. 5) and “FF_{BB}LL.B” (AAA TCC CCG GAT CCG CCA CCG CCA GAG CCA CCT CCG CCT GAA CCG CCT CCA CCT GCC) (SEQ ID NO. 6) was cloned into NaeI-SwaI-digested pXK.FF_{BB}Δ3, generating pXK.FF_{BB}LL. The duplex contains a BamHI-site

at the 3'-end of the linker-encoding sequence. Of note, this cloning procedure left both the NaeI- and the SmaI-sites intact and, therefore available for subsequent cloning steps.

An RGS(His)₆-encoding sequence was fused to the 3' end
5 of the FF_{BB}LL gene by inserting a synthetic oligo duplex made of
oligos "RGS6H.T" (GAT CTA GAG GAT CGC ATC ACC ATC ACC ATC ACT
AAT) (SEQ ID NO. 7) and "RGS6H.B" (ATT AGT GAT GGT GAT GGT GAT
GCG ATC CTC TA) (SEQ ID NO. 8) into BamHI-SmaI-digested
pXK.FF_{BB}LL. The resultant plasmid was designated pXK.FF/6H. This
10 cloning procedure destroyed both the BamHI- and the SmaI-sites.
This completed the derivation of the shuttle plasmid containing the
FF/6H gene.

In order to express the FF/6H protein in *E.coli*, the FF/6H
assembled in pXK.FF/6H was PCR amplified using the primers
15 "FF.F(BspHI) (CCC TCA TGA AGC GCG CAA GAC CGT CTG) (SEQ ID NO. 9)
and (CCC AAG CTT AGT GAT GGT GAT GGT GAT) (SEQ ID NO. 10),
digested with NcoI and HindIII and cloned into NcoI-HindIII-cut
pQE60 resulting in pQE.FF/6H.

In order to derive recombinant adenoviral genome
20 containing FF/6H gene, an EcoRI-XbaI-fragment of pXK.FF/6H was
used for recombination with SmaI-digested pVK500 (6), resulting in

pVK511. The luciferase expressing cassette was then incorporated in place of the E1 region of the adenoviral genome contained in pVK511 via homologous DNA recombination between ClaI-digested pVK511 and a fragment of pACCMV.LucΔPC. The plasmid generated was
5 designated pVK711. The virus of interest, Ad5LucFF/6H, was then rescued by transfecting 211B cells (15) with PacI-digested pVK711.

EXAMPLE 2

10

Characterization of recombinant adenovirus expressing the fiber-fibritin-6His (FF/6H) chimera

For the purposes of preliminary characterization, the FF/6H chimeric protein was initially expressed in *E.coli* and purified
15 on a Ni-NTA-agarose column. Subsequent SDS-PAGE analysis of the purified chimeric protein proved that it is trimeric and that the FF/6H trimers are as stable in an SDS-containing gel as the trimers of the wild type Ad5 fiber (Fig. 1B). Efficient binding of the FF/6H protein to a Ni-NTA-containing matrix proved that the 6His ligand
20 was available for binding in the context of this trimeric molecule. According to this analysis, truncated T4 fibrin incorporated into the

FF/6H protein was able to direct trimerization of the chimera and also successfully served the purposes of ligand presentation, thereby satisfying two key functional criteria of an ideal fiber-replacing molecule.

5 In order to evaluate the functional utility of the FF/6H chimeras incorporated into a mature adenoviral particle, homologous recombination in *E.coli* (14) was employed to insert the FF/6H encoding gene into the genome of E1-deleted, firefly luciferase-expressing Ad5 in place of the wild type fiber gene. The virus of
10 interest, Ad5LucFF/6H, was then rescued by transfection of 211B cells with the resultant adenoviral genome (Fig. 4). 211B cells, a derivative of 293 cells which constitutively express the wild type Ad5 fiber protein (15), were chosen for this transfection experiment in order to guarantee the success of the virus rescue. Ad5LucFF/6H
15 was further expanded on 211B cells and purified by double banding in a CsCl gradient. At this point, the viral stock contained mosaic virions bearing a mixture of the wild type fibers and FF/6H chimeras (data not shown). In order to obtain a homogenous population of Ad5LucFF/6H virions lacking the wild type fibers, but exclusively
20 incorporating FF/6H proteins, the original viral stock was then used to infect 293/6H cells at multiplicity of infection of 1000 viral

particles per cell. CsCl gradient purification of Ad5LucFF/6H virions isolated from the lysates of infected 293/6H cells 72 hours post infection (at which point a complete cytopathic effect was observed) resulted in a yield of 3×10^4 viral particles per cell, which was well within the range of yields characteristic for E1-deleted Ad5 vectors.

The next goal was to demonstrate that the FF/6H chimeras had been incorporated into the Ad5LucFF/6H capsids. Since fiberless Ad5 virions have been successfully purified on CsCl gradients by others (15, 16), it was possible that the putative Ad5LucFF/6H virions isolated in our study could have lacked FF/6H proteins. This was ruled out by SDS-PAGE of purified Ad5LucFF/6H virions and a Western blot analysis utilizing anti-sera specific to all three major components of FF/6H chimera, the fiber tail, the fibritin and the 6His ligand (Fig. 5A and B). These assays showed that the capsid of Ad5LucFF/6H virions consists of completely matured Ad proteins and incorporates full-size FF/6H chimeras. As expected, no wild type fibers were found in this preparation of Ad5LucFF/6H. These findings were further corroborated in an experiment involving binding of purified Ad5LucFF/6H virions to Ni-NTA-resin: in contrast to the Ad vector containing wild type fibers, which did not bind to the matrix, Ad5LucFF/6H demonstrated 6His-mediated binding to

the resin (Fig. 6). Therefore, in addition to its ability to assume a trimeric configuration and bind to a receptor-mimicking molecule, the FF/6H chimera also retained the capacity of being incorporated into mature Ad capsids.

5 Restriction enzyme analysis of the Ad5LucFF/6H genome, diagnostic PCR utilizing a pair of primers flanking the fiber gene in Ad5 genome and partial sequencing of Ad5LucFF/6H DNA demonstrated that the viral genome was stable and that the only fiber-encoding gene present was the FF/6H gene (Fig. 7). This set of
10 experiments completed the molecular characterization of Ad5LucFF/6H by confirming both the identity and the integrity of the virus capsid and its genome.

 The ability of Ad5LucFF/6H to deliver a transgene to the target cells was then evaluated in a series of studies employing this
15 viral vector for infection of 293/6H cells expressing an artificial receptor capable of binding proteins and Ad virions possessing a 6His tag (Fig. 3). First, the gene transfer capacity of Ad5LucFF/6H was compared to that of an isogenic Ad vector, Ad5Luc1, bearing wild type fibers (Fig. 8A). The doses of both viruses used in this
20 experiment were normalized based on the particle titers of the viral preparations, which also correlated well with the total protein

concentration of the samples. Due to the significant differences in the dissociation constants (k_d) previously determined for the Ad5 fiber/CAR interaction (17), 4×10^{-9} M, and for the 5His/anti-5His mAb 3D5 interaction (18), 4.75×10^{-7} M, lower efficiency of the gene transfer for Ad5LucFF/6H vector was expected.

In order to compensate for potentially lower infection levels resulting from this difference in binding affinities, several different doses of Ad5LucFF/6H vector WERE was useD, of which the lowest corresponded to the dose of the control vector. This experiment showed that Ad5LucFF/6H was capable of efficient transgene delivery to the target cells. However, at equal multiplicities of infection the level of transgene expression in Ad5Luc1-infected cells (293 and 293/6H) was 30-fold higher than that registered in 293/6H cells infected with Ad5LucFF/6H. Importantly, there was an two orders of magnitude increase in Ad5LucFF/6H-expressed luciferase activities detected in 293/6H cells expressing AR compared to parental 293 cells infected with the same vector. This differential in the transgene expression levels strongly suggests that Ad5LucFF/6H-mediated gene transfer to 293/6H occurred in a CAR-independent, receptor-specific manner via interaction of the virus with the AR.

The next gene transfer experiment employed two different forms of recombinant fibrinin proteins as blocking agents, of which only one, fibrinin-6H, contained a carboxy terminal 6His tag (Fig. 8B). The purpose of this assay was to provide additional
5 evidence that the backbone of the fibrinin molecule does not contribute to binding to AR or any other cell surface receptor. Dose-dependent inhibition of Ad5LucFF/6H infection of 293/6H cells with fibrinin-6H, but not with the fibrinin lacking the 6His tag, further proved that this tag is the component of the virion solely responsible
10 for the binding of the virus to the AR.

The present invention has developed a novel approach to the modification of adenoviral vector tropism by replacing the receptor-binding fiber protein in the adenoviral capsid with an artificial protein chimera. The rational design of this chimera, based
15 on the general structural similarity of the Ad5 fiber and bacteriophage T4 fibrinin, has resulted in the derivation of a novel ligand-presenting molecule. The most important difference from the wild type fiber protein is the disengagement of the trimerization and the receptor-binding functions normally performed by the fiber
20 knob domain. As a result of this distribution of functions, the receptor specificity of the re-engineered Ad5 vector may now be

defined by a domain of the chimera which plays no role in the trimerization of the molecule, and may therefore be manipulated without the risk of destabilizing the ligand-presenting protein and the virion. The use of T4 fibrin for ligand display suggests that a
5 wide variety of heterologous targeting ligands, including large polypeptide molecules, may be employed in the context of the fiber-fibrin chimera described here.

Fibrin chimeras analogous to the one described in this work may be viewed as versatile ligand-displaying molecules
10 suitable for genetic modification of virtually any human or animal adenoviral vector. The problem of elimination of undesirable natural tropism of native fibers contained in the adenoviral virion may thus be solved by substitution of native fibers with such fibrin chimeras. This approach has significant advantage over maneuvers involving
15 the identification and subsequent mutagenesis of the native receptor binding sites within the fibers of numerous adenoviral species, some of which are able to bind to different types of primary receptors. In addition, this strategy eliminates the risk of reversion of the mutated fiber gene to the wild type during multiple rounds of propagation,
20 which would compromise the efficiency of any vector targeting schema.

An additional advantage offered by adenoviral vectors incorporating the fibritin-based chimeras for the purposes of human gene therapy because of interference of anti-fiber antibodies present in the serum of some gene therapy patients with the adenoviral
5 vectors used in clinical protocols. Importantly, these antibodies have been shown to have a synergistic effect on adenoviral vector neutralization when present together with anti-penton base antibodies. Thus, deletion of the most of the fiber sequence in the fibritin-bearing adenoviral vectors would make them refractory to
10 this type of immune response and therefore more efficient as therapeutic agents.

EXAMPLE 3

15

Characterization of recombinant adenovirus expressing the fiber-fibritin-RGD-6His (FF.RGD/6H) chimera

A second adenoviral vector, Ad5luc.FF.RGD/6H, containing fiber-fibritin chimeras incorporating at their carboxy termini two
20 peptide ligands RGD-4C (CDCRGDCFC) (SEQ ID NO. 14) and 6His was

generated (Fig. 9). The virus was propagated in 293 cells and purified on CsCl gradient according to standard technique.

The protein composition of Ad5luc.FF.RGD/6H was verified by SDS-PAGE using the virus with wild type capsids as a control. As shown in Figure 10, all major protein components of Ad5luc.FF.RGD/6H are essentially the same as those of control adenoviral capsid. The only difference noted between the capsid protein patterns demonstrated by the two viruses was the presence of the FF.RGD/6H chimeras in the Ad5LucFF.RGD/6H particles in place of the wild type fibers contained in the capsids of the control adenovirus.

FF.RGD/6H chimeras present in the preparation of Ad5luc.FF.RGD/6H were further identified by Western blot analysis utilizing a set of antibodies specific to each of the component of the chimeric protein. The presence of the fiber tail domain, the fibritin fragment and the 6His tag was confirmed by using relevant mono- and polyclonal antibodies (Fig. 11).

Association of the FF.RGD/6H chimeras with the Ad5luc.FF.RGD/6H particles was proved by incubating purified Ad5luc.FF.RGD/6H virions with Ni-NTA-sepharose which is designed for purification of the 6His-tagged proteins. In contrast to control

adenoviral vector containing wild type fibers which did not bind to Ni-NTA, Ad5luc.FF.RGD/6H was efficiently retained on the column. The presence of all major adenoviral capsid proteins in the material eluted from the resin with imidazole suggested that the
5 Ad5luc.FF.RGD/6H virions were anchored to Ni-NTA-sepharose by virtue of the 6His-containing fiber-fibritin chimeras associated with the virions (Fig. 12).

In order to rule out the possibility of contamination of Ad5luc.FF.RGD/6H preparation with another adenoviral vector,
10 Ad5luc.FF.RGD/6H DNA isolated from virions was subjected to three different assay including restriction enzyme analysis (Fig. 13), “diagnostic” PCR, and sequencing of the fiber-fibritin gene as well as the regions of Ad genome adjacent to it. All three assays showed that the preparation of Ad5luc.FF.RGD/6H is free from any
15 contaminating adenovirus and therefore is suitable for subsequent studies aimed to characterize the gene transfer capacity and the cell entry pathway utilized by Ad5luc.FF.RGD/6H.

To evaluate the gene transfer capacity of Ad5luc.FF.RGD/6H, the virus was employed for gene delivery
20 experiments utilizing two different cell lines: 293 and 293/6H. The latter of the two lines is the derivative of 293 cells constitutively

expressing artificial receptor capable of binding 6His-tagged proteins.

The luciferase-expressing adenoviral vector isogenic to Ad5luc.FF.RGD/6H but incorporating the wild type fibers was used in these experiments as a control. The gene transfer with the control virus was done at one multiplicity of infection (MOI), whereas Ad5luc.FF.RGD/6H was used at different MOIs.

As shown in Figure 14, Ad5luc.FF.RGD/6H can deliver a luciferase reporter to both types of cells, although with rather different efficiencies (luciferase expression in naïve 293 cells was always lower than in 293/6H cells), thereby suggesting that both the RGD-4C and the 6His peptides incorporated within the FF.RGD/6H chimeras functioned as targeting ligands.

The following references were cited herein:

1. Bergelson et al., *Science* **275**, 1320-3 (1997).
2. Tomko et al., *Proc. Natl. Acad. Sci.* **94**, 3352-6 (1997).
3. Krasnykh et al., *Molecular Therapy* **1**, 391-405 (2000).
4. Wickham et al., *Nat Biotechnol* **14**, 1570-3 (1996).
5. Wickham et al., *Journal of Virology* **71**, 8221-8229 (1997).
6. Dmitriev et al., *J Virol* **72**, 9706-13 (1998).
7. Vanderkwaak et al., *Gynecol Oncol* **74**, 227-34 (1999).
8. Kasono et al., *Clinical cancer research* **5**, 2571-2579 (1999).

9. Hong and Engler, *J Virol* **70**, 7071-8 (1996).
10. Tao et al., *Structure* **5**, 789-98 (1997).
11. Letarov et al., *Biochemistry (Mosc)* **64**, 817-23 (1999).
12. V. V. Mesyanzhinov, personal communication.
- 5 13. Douglas et al., *Nat Biotechnol* **17**, 470-5 (1999).
14. Krasnykh et al., *J Virol* **72**, 1844-52 (1998).
15. Von Seggern et al., *J Gen Virol* **79**, 1461-8 (1998).
16. Legrand et al., *J Virol* **73**, 907-19 (1999).
17. Davison et al., *J Virol* **73**, 4513-4517 (1999).
- 10 18. Lindner et al., *Biotechniques* **22**, 140-9 (1997).
19. Miroshnikov et al., *Protein Eng* **11**, 329-32 (1998).
20. Efimov et al., *Virus Genes* **10**, 173-7 (1995).
21. H. Gahery-Segard, et al., *J Virol* **72**, 2388-97 (1998).
22. Krasnykh et al., *J. Virol.* **70**:6839-46 (1996).
- 15 Any patents or publications mentioned in this
specification are indicative of the levels of those skilled in the art to
which the invention pertains. These patents and publications are
herein incorporate by reference to the same extent as if each
individual publication was specifically and individually indicated to
20 be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, an specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED:

1. An adenovirus with novel vector tropism and ablation of native adenoviral receptor tropism due to the expression
5 of a fiber replacement protein, said fiber replacement protein comprises:

a) an amino-terminal portion comprising an adenoviral fiber tail domain;

b) a chimeric protein that provides trimerization
10 function; and

c) a carboxy-terminal portion comprising a targeting ligand.

15 2. The adenovirus of claim 1, wherein said fiber replacement protein associates with the penton base of the adenovirus.

20 3. The adenovirus of claim 1, wherein said fiber replacement protein is a rod-like, trimeric protein.

4. The adenovirus of claim 3, wherein said rod-like, trimeric protein has a diameter comparable to the native fiber protein of wild type adenovirus.

5

5. The adenovirus of claim 1, wherein said fiber replacement protein retains trimerism when a sequence encoding a targeting ligand is incorporated into the carboxy-terminus.

10

6. The adenovirus of claim 1, wherein said fiber replacement protein is soluble.

15

7. The adenovirus of claim 1, wherein said fiber replacement protein is T4 bacteriophage fibritin protein

20

8. The adenovirus of claim 1, wherein said fiber replacement protein is selected from the group consisting of trimeric

structural proteins, trimeric viral proteins and trimeric transcription factors.

5 9. The adenovirus of claim 1, wherein said fiber replacement protein contains isoleucine trimerization motif.

10 10. The adenovirus of claim 1, wherein said fiber replacement protein is neck region peptide from human lung surfactant D.

15 11. The adenovirus of claim 1, wherein said fiber replacement protein is an artificial protein having a coiled coil secondary structure, wherein said secondary structure is stable because of multiple interchain interactions.

20 12. The adenovirus of claim 1, wherein said targeting ligand is selected from the group consisting of physiological ligands,

anti-receptor antibodies, cell-specific peptides and single chain antibodies.

5 13. The adenovirus of claim 1, wherein said adenovirus carries in its genome a therapeutic gene.

10 14. The adenovirus of claim 13, wherein said therapeutic gene is a herpes simplex virus thymidine kinase gene.

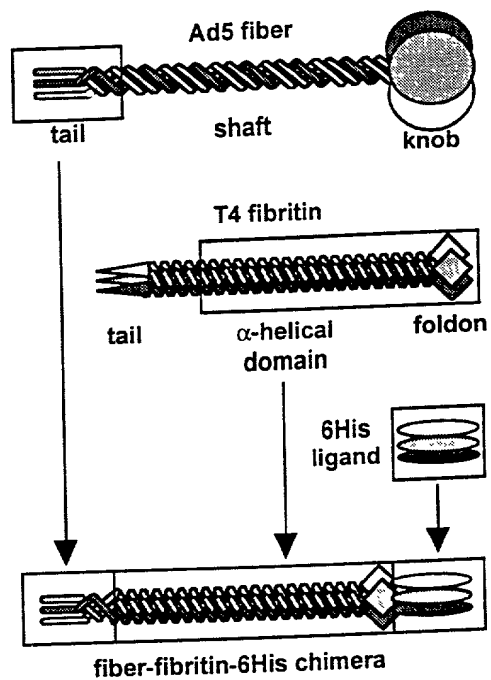
15 15. A method of killing tumor cells in an individual in need of such treatment, comprising the steps of:
 pretreating said individual with an effective amount of the adenovirus of claim 14; and
 administering ganciclovir to said individual.

ABSTRACT OF THE DISCLOSURE

The utility of adenovirus vectors (Ad) for gene therapy is
5 restricted by their inability to selectively transduce disease-affected
tissues. This limitation may be overcome by the derivation of
vectors capable of interacting with receptors specifically expressed in
the target tissue. Previous attempts to alter Ad tropism by genetic
modification of the Ad fiber have had limited success due to
10 structural conflicts between the fiber and the targeting ligand. The
present invention presents a strategy to derive an Ad vector with
enhanced targeting potential by a radical replacement of the fiber
protein in the Ad capsid with a chimeric molecule containing a
heterologous trimerization motif and a receptor-binding ligand.

15

A



B

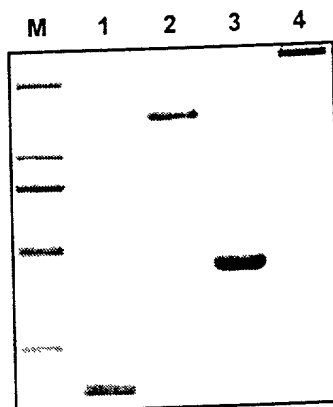


Fig. 1

fiber tail

MKRARPS EDTFNPVYPYDTETGPPTVPFLT PPFVSPNGFQESPP

1st repeat of the fiber shaft

2nd repeat of the fiber shaft

fiber/fibrin junction

GVLSRLSEPLVTSN

GMALKMGNGLSLDEA

GNLT**SQNVYSRLNEI**

7th coiled coil of the fibrin

DTKQTTVESDISAIKTSI

GYPGNN

SIITSVNTNTDNIA SINLEL

NQSGG

8th coiled coil of the fibrin

9th coiled coil of the fibrin

IKQRLTVIETSI

GSDDIPSS

IKGQIKDNTTSIESLNGIV

GENTSSG

LRA

10th coiled coil of the fibrin

11th coiled coil of the fibrin

NVSWLNQIV

GTDSSGGQPSPPG

SLLNRVSTIETSVSGLNNDVQNL

12th coiled coil of the fibrin

QVEI

GNNSTG

IKGQVVALNTLV

NGTNPNGSTVEERG

LTNSIKANET

13th coiled coil of the fibrin

trimerization domain of the fibrin

NIASVTQEVNTAKGNISLQGDVQALQEA

GYIPEAPRDGQAYVRK

linker

6His ligand

DGEWLLSTFLSPA

GGGGSGGGSGGGGS

RGSHHHHHH

(Seq ID. No. 13)

Fig. 2

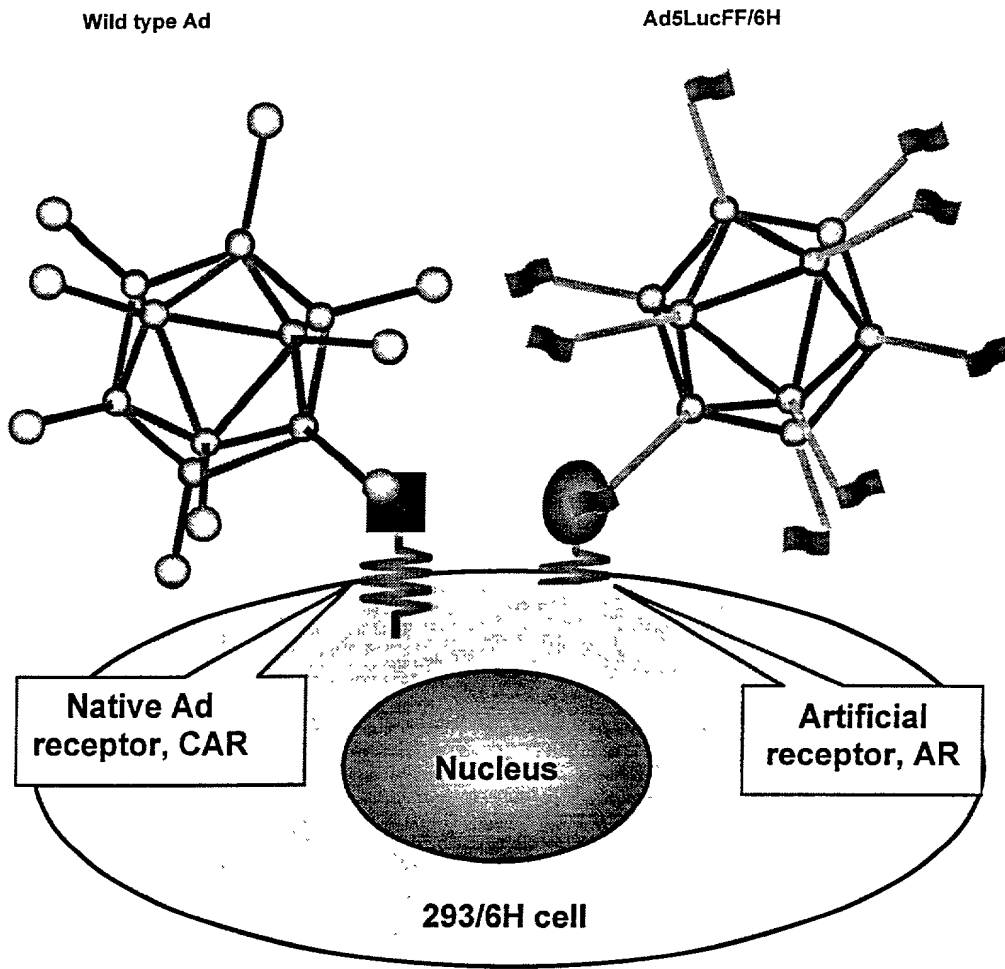


Fig. 3

**Ad5LucFF/6H genome
generated in *E. coli***

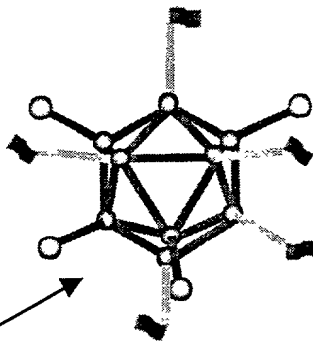


**Transfection
of 211B cells**



**Formation
of plaques**

**Virions with
mosaic capsids**



Infection of 293/6H cells



**Ad5LucFF/6H
virions containing
FF/6H chimeras**

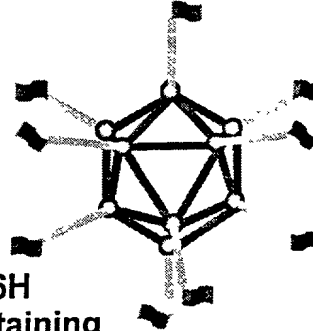
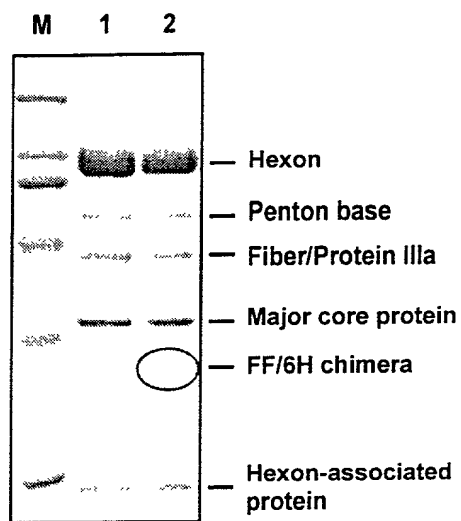


Fig. 4

A



B

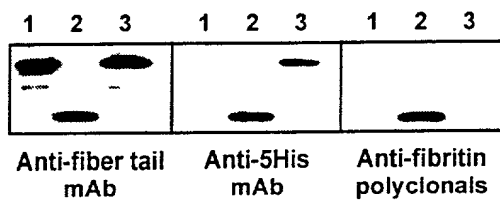


Fig. 5

A

| M | Wild type Ad5 | | | | Ad5LucFF/6H | | | | M | |
|---|---------------|---|---|---|-------------|---|---|---|---|--|
| | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | | |
| | | | | | | | | | | |

B

| | |
|--|--|
| | |
|--|--|

C

| | |
|--|--|
| | |
|--|--|

Fig. 6

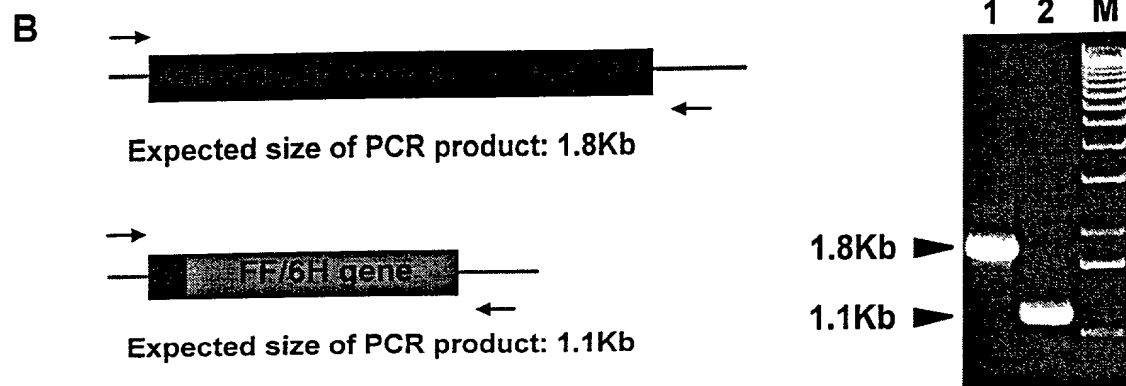
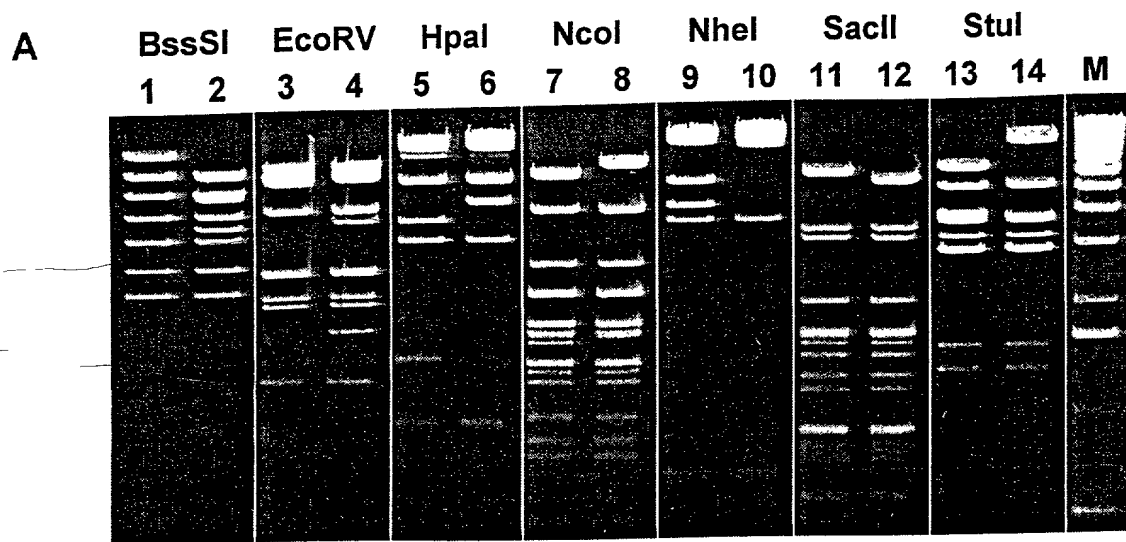
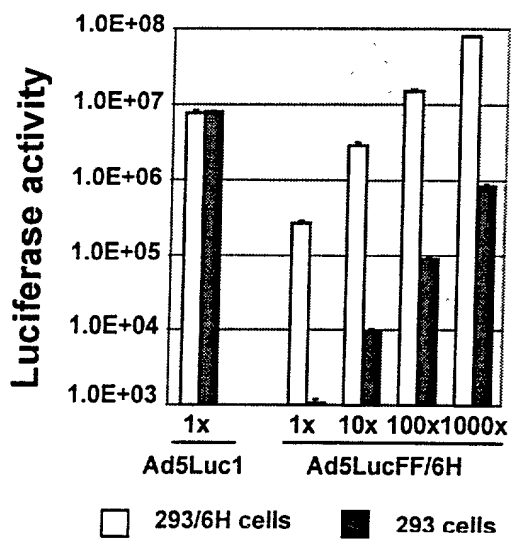


Fig. 7

A



B

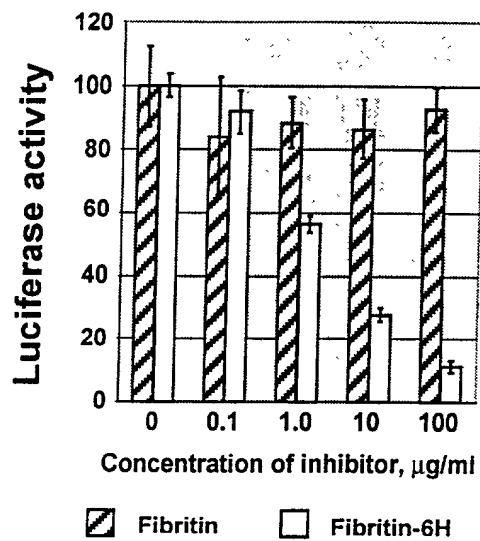


Fig. 8

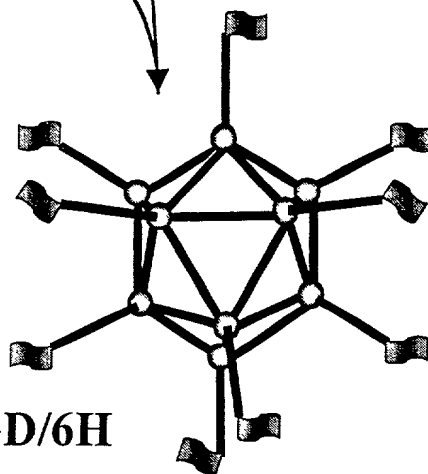
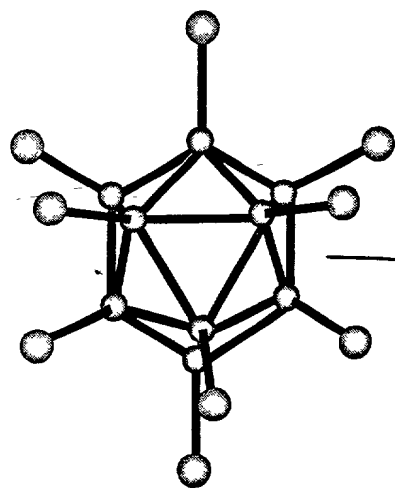
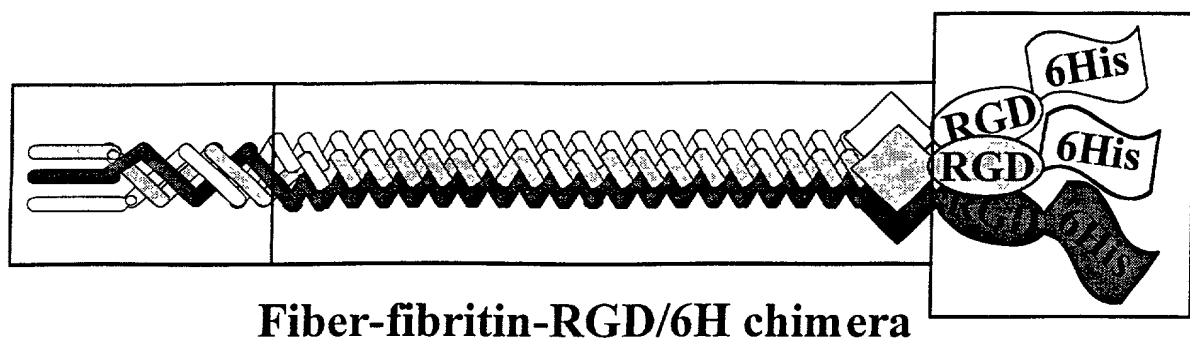


Fig. 9

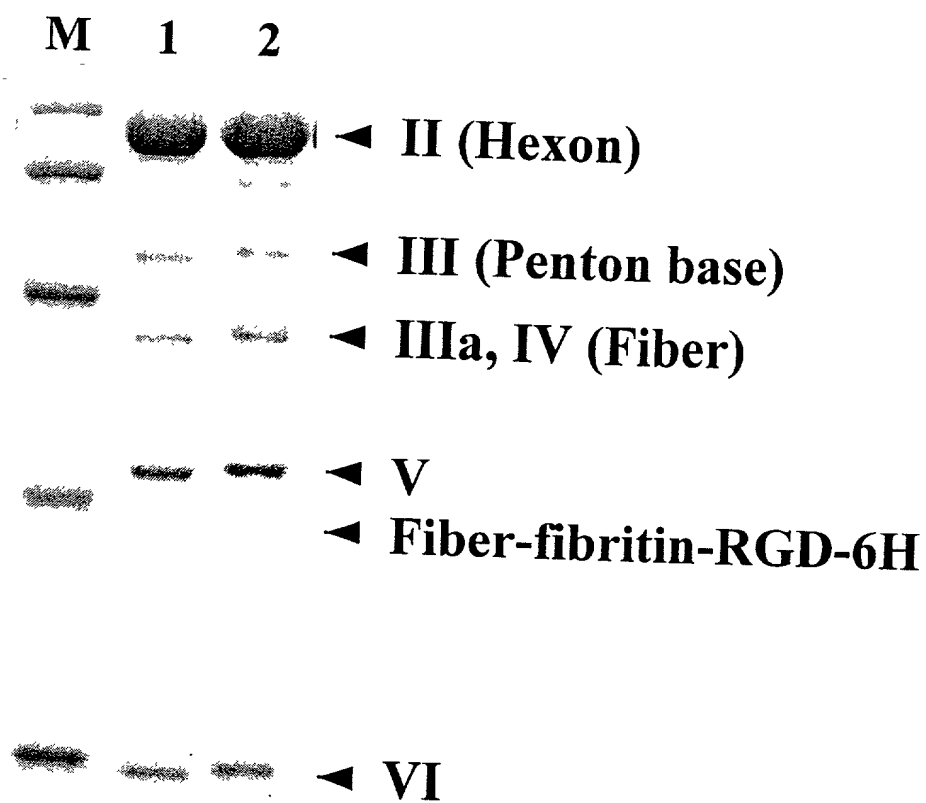


Fig. 10

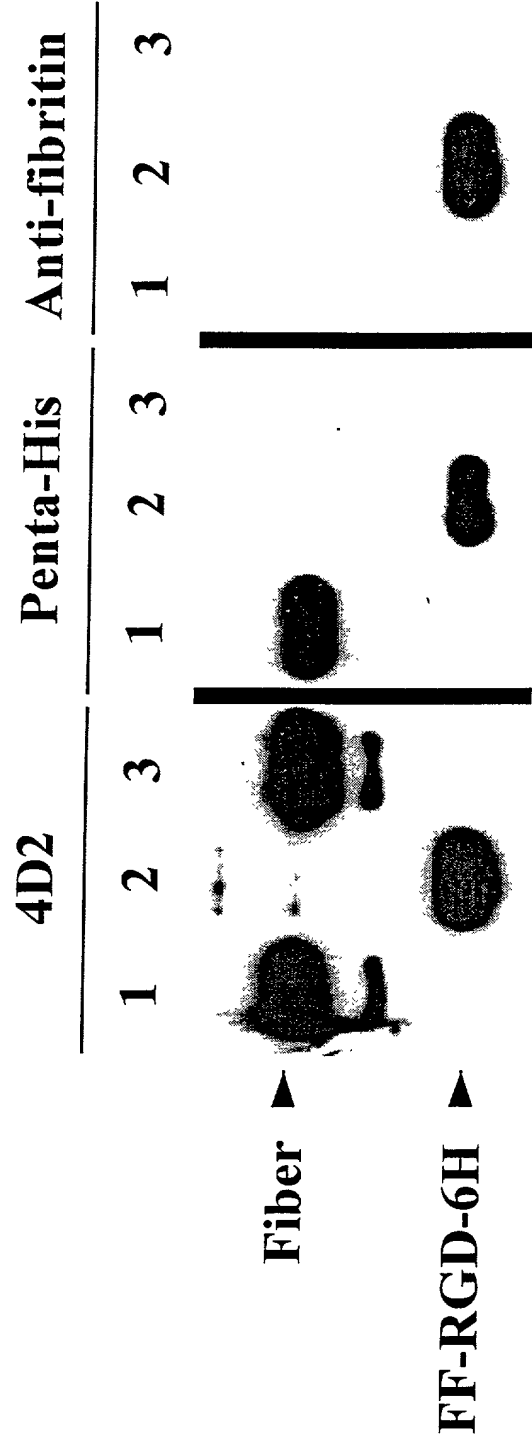


Fig. 11

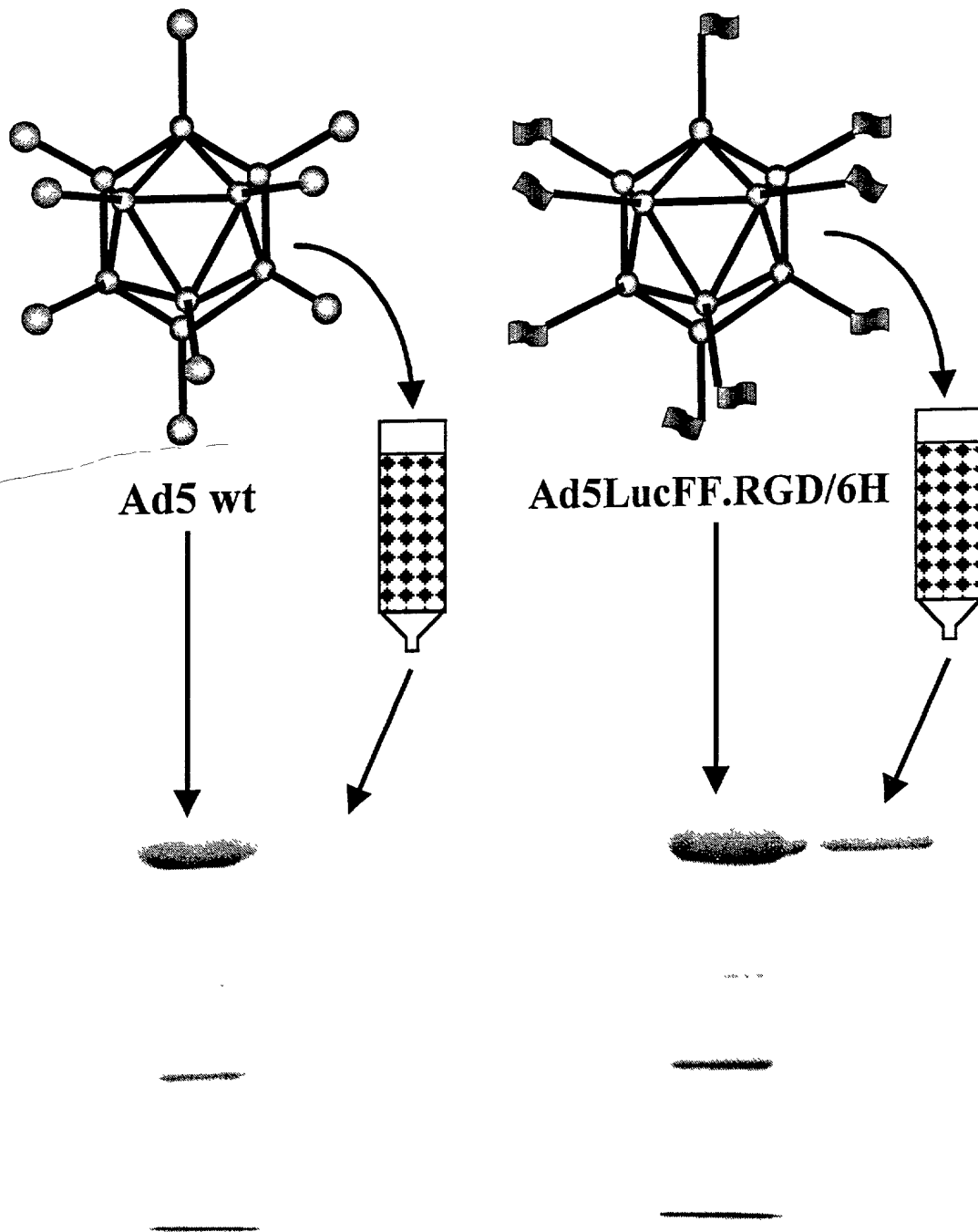
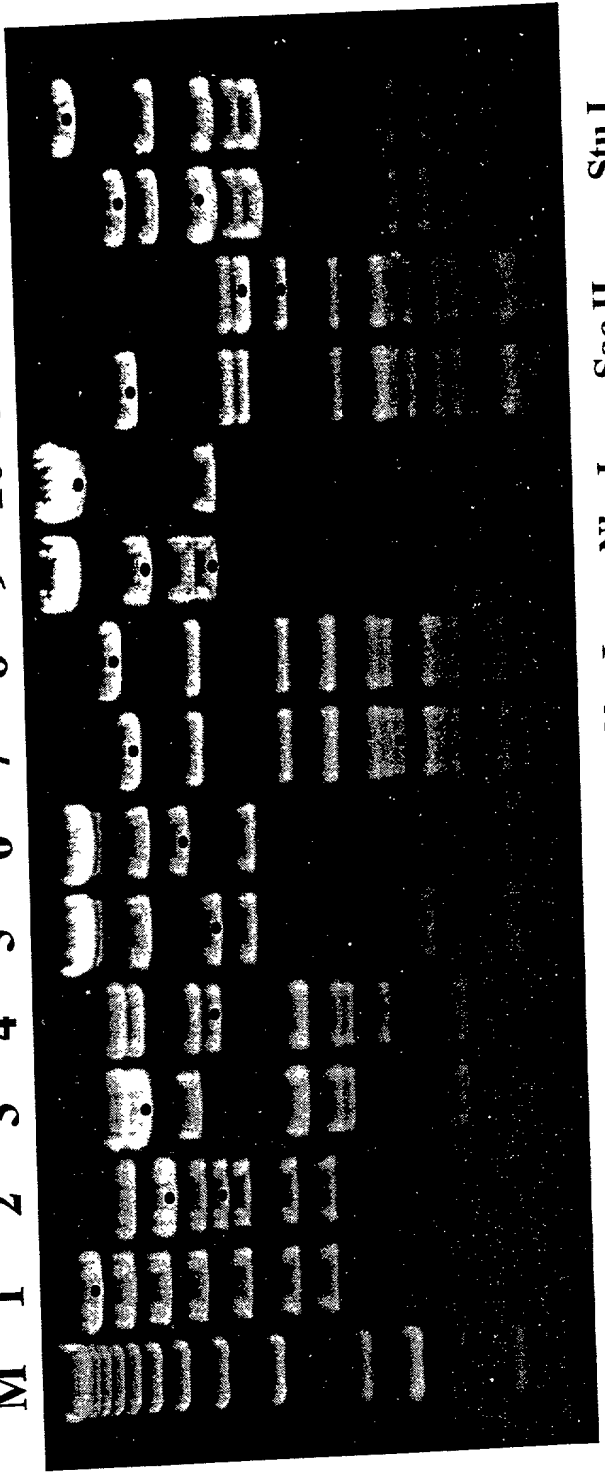


Fig. 12

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14

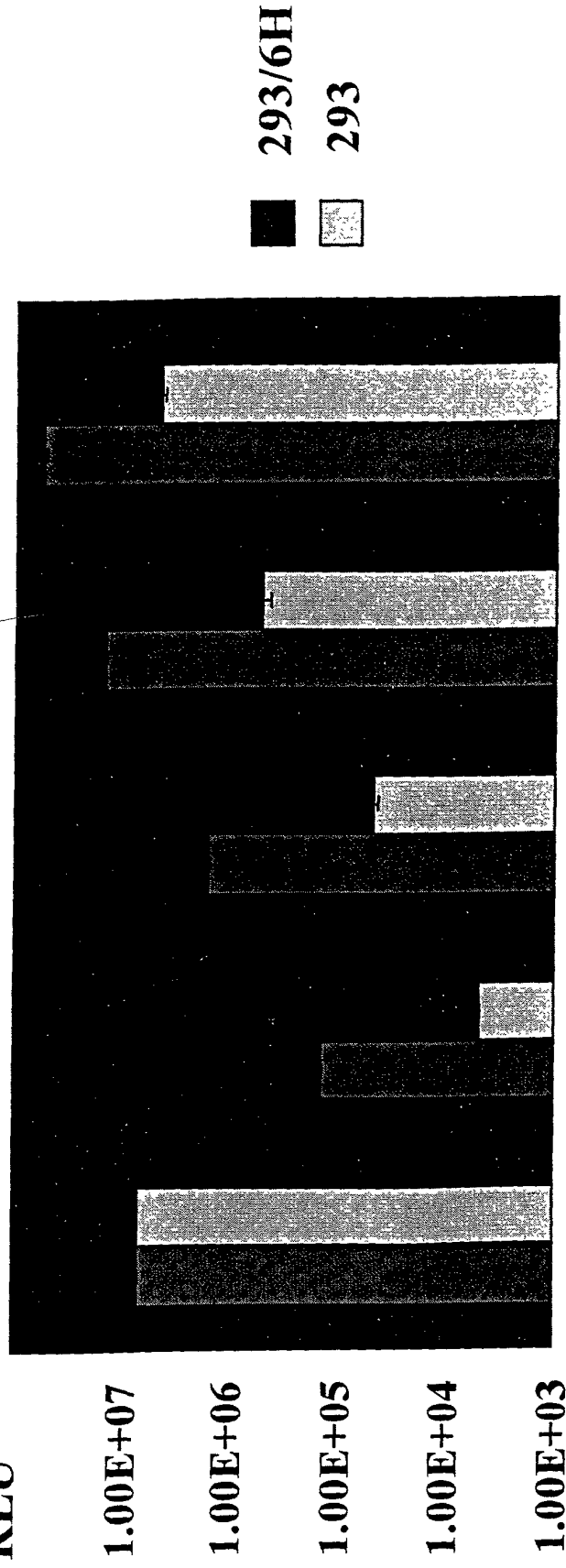


BssSI EcoRV HpaI NcoI NheI SacII StuI

Fig. 13

10000000
 1000000
 100000
 10000
 1000
 100
 10
 1

Luciferase activity,
 RLU



| Virus | Ad5Luc1 | Ad5.FF.RGD/6H |
|-------|-------------------|-------------------|
| Dose | 1x 10x 100x 1000x | 1x 10x 100x 1000x |

Fig. 14

FROM : MCGREGOR&ADLER, P.C.

PHONE NO. :

Jul. 05 2000 05:51PM P2

DOCKET NO: D6070CIP

COMBINED DECLARATION AND POWER OF ATTORNEY

I, Victor N. Krasnykh, hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and joint inventor, together with David T. Curiel, of the subject matter which is claimed and for which a patent is sought on the invention entitled, **Modified Adenovirus Containing a Fiber Replacement Protein**; the specification of which is attached hereto and claims benefit of priority of US application 09/250,580 filed February 16, 1999, which claims benefit of priority under 35 U.S.C. 119(e) of provisional application USSN 60/074,844 filed February 17, 1998, now abandoned.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.55(a).

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Dr. Benjamin Adler, Registration No. 35,423. Address all telephone calls to Dr. Benjamin Adler at telephone number 713/777-2321. Address correspondence to Dr. Benjamin Adler, MCGREGOR & ADLER, LLP, 8011 Candle Lane, Houston, TX 77071.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patents issued thereon.

Full Name of Inventor: Victor N. KrasnykhInventor's Signature: Victor Krasnykh Date: 07-06-2000Residence Address: 6914 Rime Village Drive, Birmingham AL 35216Citizen of: RussiaPost Office Address: 6914 Rime Village Drive, Birmingham, AL 35216

Jul 06 00 12:09p

Divison of Gene Therapy

+31 20 444 8168

p. 15

FROM : MCGREGOR&ADLER, P.C.

PHONE NO. :

Jul. 05 2000 05:58PM P2

DOCKET NO: D6070CIP

COMBINED DECLARATION AND POWER OF ATTORNEY

I, David T. Curiet, hereby declare that:

My residence, post office address and citizenship are as stated below next to my name. I believe I am the original first and joint inventor, together with Victor N. Krasnykh, of the subject matter which is claimed and for which a patent is sought on the invention entitled, **Modified Adenovirus Containing a Fiber Replacement Protein**; the specification of which is attached hereto and claims benefit of priority of US application 09/250,580 filed February 16, 1999, which claims benefit of priority under 35 U.S.C. 119(c) of provisional application USSN 60/074,844 filed February 17, 1998, now abandoned.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Dr. Benjamin Adler, Registration No. 35,423. Address all telephone calls to Dr. Benjamin Adler at telephone number 713/777-2321. Address correspondence to Dr. Benjamin Adler, MCGREGOR & ADLER, LLP, 8011 Candle Lane, Houston, TX 77071.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Full Name of Inventor: David T. CurietInventor's Signature:  Date: 7/6/00Residence Address: 824 Linwood Dr., Birmingham, AL 35222Citizen of: United States of AmericaPost Office Address: 824 Linwood Dr., Birmingham, AL 35222

SEQUENCE LISTING

<110> Curiel, David T.
Krasnykh, Victor N.

<120> Modified Adenovirus Containing A Fiber
Replacement Protein

<130> D6070CIP

<141> 2000-07-10

<150> US 09/250,580

US 60/074,844

<151> 1999-02-16

1998-02-17

<160> 14

<210> 1

<211> 40

<212> DNA

<213> artificial sequence

<220>

<221> primer_bind

<223> Forward primer FF.F used to amplify segment of the T4
fibrin gene encoding amino acids Ser-229 through
the carboxy terminal Ala-487.

<400> 1

gggaacttga cctcacagaa cgtttatagt cgtttaaagt 40

<210> 2

<211> 37

<212> DNA

<213> artificial sequence

<220>

<221> primer_bind

<223> Reverse primer FF.R used to amplify segment of the T4
fibrin gene encoding amino acids Ser-229 through
the carboxy terminal Ala-487.

<400> 2

aggccatggc caatttttgc cggcgataaa aaggtag 37

<210> 3
 <211> 53
 <212> DNA
 <213> artificial sequence
 <220>
 <221>
 <223> synthetic oligo, F5.Δ3Swa.T, for the introduction of
 SwaI restriction site
 <400> 3

ttggcccat ttaaataaat cgtttggtt atgtttcaac gtgtttattt ttc 53

<210> 4
 <211> 61
 <212> DNA
 <213> artificial sequence
 <220>
 <221>
 <223> synthetic oligo, F5.Δ3Swa.B, for the introduction of
 SwaI restriction site
 <400> 4

aattgaaaaa taaacacgtt gaaacataac acaaacgatt catttaaattg 50
 gggccaatat t 61

<210> 5
 <211> 57
 <212> DNA
 <213> artificial sequence
 <220>
 <221>
 <223> synthetic oligo, FF_{BB}LL.T
 <400> 5

ggcaggtgga ggcggttcag gcggaggtgg ctctggcggg ggcggatccg 50
 gggattt 57

<210> 6

<211> 57
 <212> DNA
 <213> artificial sequence
 <220>
 <221>
 <223> synthetic oligo, FF_{BB}LL.B
 <400> 6

aaatccccgg atccgccacc gccagagcca cctccgcctg aaccgcctcc 50
 acctgcc 57

<210> 7
 <211> 36
 <212> DNA
 <213> artificial sequence
 <220>
 <221>
 <223> synthetic oligo, RGS6H.T
 <400> 7

gatctagagg atcgcatcac catcaccatc actaat 36

<210> 8
 <211> 32
 <212> DNA
 <213> artificial sequence
 <220>
 <221>
 <223> synthetic oligo, RGS6H.B
 <400> 8

attagtgatg gtgatggtga tgcgacctc ta 32

<210> 9
 <211> 27
 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 <223> primer to PCR amplify FF/6H in pXK.FF/6H

<400> 9
ccctcatgaa gcgcgcaaga ccgtctg 27

<210> 10
<211> 27
<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
<223> primer to PCR amplify FF/6H in pXK.FF/6H
<400> 10

cccaagctta gtgatggtga tggatgat 27

<210> 11
<211> 8
<212> PRT
<213> Adenovirus type 5
<220>
<221> DOMAIN
<223> the beginning of the third pseudorepeat of the
fiber shaft domain
<400> 11

Gly Asn Thr Leu Ser Gln Asn Val
5 8

<210> 12
<211> 26
<212> PRT
<213> Phage T4
<220>
<221> DOMAIN
<223> the sixth coiled coil segment of the α -helical
central domain of the fibritin
<400> 12

Val Tyr Ser Arg Leu Asn Glu Ile Asp Thr Lys Gln Thr Thr Val
5 10 15

Glu Ser Asp Ile Ser Ala Ile Lys Thr Ser Ile
 20 25

<210> 13
 <211> 361
 <212> PRT
 <213> artificial sequence
 <220>
 <221> CHAIN
 <223> the fiber-fibritin-6H chimera
 <400> 13

| | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Lys | Arg | Ala | Arg | Pro | Ser | Glu | Asp | Thr | Phe | Asn | Pro | Val | Tyr | 5 | 10 | 15 |
| Pro | Tyr | Asp | Thr | Glu | Thr | Gly | Pro | Pro | Thr | Val | Pro | Phe | Leu | Thr | 20 | 25 | 30 |
| Pro | Pro | Phe | Val | Ser | Pro | Asn | Gly | Phe | Gln | Glu | Ser | Pro | Pro | Gly | 35 | 40 | 45 |
| Val | Leu | Ser | Leu | Arg | Leu | Ser | Glu | Pro | Leu | Val | Thr | Ser | Asn | Gly | 50 | 55 | 60 |
| Met | Ala | Leu | Lys | Met | Gly | Asn | Gly | Leu | Ser | Leu | Asp | Glu | Ala | Gly | 65 | 70 | 75 |
| Asn | Leu | Thr | Ser | Gln | Asn | Val | Tyr | Ser | Arg | Leu | Asn | Glu | Ile | Asp | 80 | 85 | 90 |
| Thr | Lys | Gln | Thr | Thr | Val | Glu | Ser | Asp | Ile | Ser | Ala | Ile | Lys | Thr | 95 | 100 | 105 |
| Ser | Ile | Gly | Tyr | Pro | Gly | Asn | Asn | Ser | Ile | Ile | Thr | Ser | Val | Asn | 110 | 115 | 120 |
| Thr | Asn | Thr | Asp | Asn | Ile | Ala | Ser | Ile | Asn | Leu | Glu | Leu | Asn | Gln | 125 | 130 | 135 |
| Ser | Gly | Gly | Ile | Lys | Gln | Arg | Leu | Thr | Val | Ile | Glu | Thr | Ser | Ile | 140 | 145 | 150 |
| Gly | Ser | Asp | Asp | Ile | Pro | Ser | Ser | Ile | Lys | Gly | Gln | Ile | Lys | Asp | 155 | 160 | 165 |
| Asn | Thr | Thr | Ser | Ile | Glu | Ser | Leu | Asn | Gly | Ile | Val | Gly | Glu | Asn | 170 | 175 | 180 |
| Thr | Ser | Ser | Gly | Leu | Arg | Ala | Asn | Val | Ser | Trp | Leu | Asn | Gln | Ile | | | |

| | | | |
|---|-----|-----|-----|
| | 185 | 190 | 195 |
| Val Gly Thr Asp Ser Ser Gly Gly Gln Pro Ser Pro Pro Gly Ser | | | |
| | 200 | 205 | 210 |
| Leu Leu Asn Arg Val Ser Thr Ile Glu Thr Ser Val Ser Gly Leu | | | |
| | 215 | 220 | 225 |
| Asn Asn Asp Val Gln Asn Leu Gln Val Glu Ile Gly Asn Asn Ser | | | |
| | 230 | 235 | 240 |
| Thr Gly Ile Lys Gly Gln Val Val Ala Leu Asn Thr Leu Val Asn | | | |
| | 245 | 250 | 255 |
| Gly Thr Asn Pro Asn Gly Ser Thr Val Glu Glu Arg Gly Leu Thr | | | |
| | 260 | 265 | 270 |
| Asn Ser Ile Lys Ala Asn Glu Thr Asn Ile Ala Ser Val Thr Gln | | | |
| | 275 | 280 | 285 |
| Glu Val Asn Thr Ala Lys Gly Asn Ile Ser Ser Leu Gln Gly Asp | | | |
| | 290 | 295 | 300 |
| Val Gln Ala Leu Gln Glu Ala Gly Tyr Ile Pro Glu Ala Pro Arg | | | |
| | 305 | 310 | 315 |
| Asp Gly Gln Ala Tyr Val Arg Lys Asp Gly Glu Trp Val Leu Leu | | | |
| | 320 | 325 | 330 |
| Ser Thr Phe Leu Ser Pro Ala Gly Gly Gly Gly Ser Gly Gly Gly | | | |
| | 335 | 340 | 345 |
| Gly Ser Gly Gly Gly Gly Ser Arg Gly Ser His His His His His | | | |
| | 350 | 355 | 360 |
| His | | | |
| 361 | | | |

| | |
|-------|---|
| <210> | 14 |
| <211> | 9 |
| <212> | PRT |
| <213> | Unknown |
| <220> | |
| <221> | DOMAIN |
| <223> | a peptide ligand containing the RGD motif |
| <400> | 14 |

Cys Asp Cys Arg Gly Asp Cys Phe Cys

5

9

SEQ 6